Regulation of the Actin Cytoskeleton by Twinfilin

Sandra Falck

Institute of Biotechnology
Department of Biological and Environmental Sciences
Division of Genetics
Faculty of Biosciences and
Viikki Graduate School in Biosciences
University of Helsinki

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Supervised by

Docent Pekka Lappalainen
Institute of Biotechnology
University of Helsinki

Reviewed by

Docent Olli Carpén
Department of Anatomy
University of Helsinki

and

Docent Jussi Jäntti
VTT Biotechnology

Opponent

Professor Roger Karlsson
Department of Cell Biology
Stockholm University, Sweden

Cover figure: Calcofluor staining illustrating the random budding pattern of *Dtwf1/Dtwf1* yeast cells.

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To my family

Contents

ABBREVIATIONS

Abp1	actin binding protein 1
ADP	adenosine diphosphate
ADF	actin depolymerizing factor
ADF-H	actin depolymerizing factor-homology
Aip1	actin interacting protein 1
Arp	actin related protein
ATP	adenosine triphosphate
CAP	cyclase-associated protein
Cc	critical concentration
CH	calponin homology
CRIB	Cdc42/Rac interactive binding
DAG	diacylglycerol
F-actin	filamentous actin
FH	formin homology
G-actin	globular actin, monomeric actin
GAP	GTPase activating protein
GBD	GTPase binding domain
GDI	guanosine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanosine nucleotide exchange factor
GFP	green fluorescent protein
GST	gluthatione S-transferase
GTP	guanosine triphosphate
IP_3	inositol 1,4,5-triphosphate
NBD	7-chloro-4-nitrobenz-2-oxa-1,3-diazole
NPF	nucleation promoting factor
N-WASP	neural WASP
PI	phosphoinositide
PKC	protein kinase C
RNP	ribonucleoprotein particle
Scar	suppressor of cAMP receptor mutation
SH	Src homology
WASp	Wiscott-Aldrich syndrome protein
WAVE	WASP verprolin homologous protein
WH	WASP homology
WIP	WASP-interacting protein

ORIGINAL PUBLICATIONS

This thesis is based on three original articles, and one review article, which are referred to in the text by their Roman numerals.

I **Palmgren S**, Ojala PJ, Wear MA, Cooper JA, Lappalainen P (2001): Interactions with PIP2, ADP-actin monomers and capping protein regulate the activity and localization of yeast twinfilin. J. Cell Biol. 155: 251-260.

II Paavilainen VO, Merckel, CM, **Falck S**, Ojala PJ, Pohl E, Wilmanns M, Lappalainen P (2002): Structural conservation between the actin monomer-binding sites of ADF/cofilin and twinfilin. J. Biol. Chem. 277: 43089-43095.

III **Falck S**, Paavilainen VO, Wear MA, Grossmann GJ, Cooper JA, Lappalainen P (2004): Biological role and structural mechanism of twinfilin-capping protein interaction. (submitted)

IV **Palmgren S**, Vartiainen MK, Lappalainen P (2002): Twinfilin, a molecular mailman for actin monomers. J.Cell Sci. 115: 881-886. (Review article)

SUMMARY

As the most abundant protein in the majority of eukaryotic cells, actin is involved in a wide variety of cellular processes. In comparison to the stable actin structures present in muscle cell, non-muscle cell actin is very dynamic and the filamentous structures it forms are regulated by a large number of actin binding proteins. Numerous proteins bind actin in its monomeric form, and a central task of these proteins is to regulate the size and localization of the cellular actin monomer pool. A pool of free actin monomers is needed to enable actin filament polymerization to rapidly take place in response to the changing needs of the cell.

Twinfilin is a small ubiquitous protein that shows sequence homology to one of the actin binding proteins, ADF/cofilin. Originally identified from yeast, twinfilin binds actin monomers in a 1:1 complex. As a recently identified actin monomer binding protein, only the basic biochemical characteristics of twinfilin were previously described, and a deeper knowledge of this protein was needed to gain understanding of its role in actin dynamics. In this PhD-thesis work, biochemical, cell biological, and yeast genetics methods have been used to investigate the role of twinfilin in actin dynamics. We found twinfilin to be an abundant protein that localizes to the cortical actin patches in yeast cells. We also discovered that twinfilin preferentially binds monomeric actin in its ADP-bound form. Furthermore, our studies demonstrated twinfilin to directly interact with capping protein at the barbed end of actin filaments and to bind and be inactivated by $PI(4,5)P_2$. In addition, we found that correct localization of twinfilin in cells is dependent on interaction with both an ADP-actin monomer as well as with capping protein. By mutagenesis studies we mapped the binding sites for actin and capping protein on the twinfilin molecule. Using specific yeast twinfilin mutants we showed that twinfilin's interaction with capping protein is essential for its role in actin filament turnover *in vivo*.

Taken together, this data brings us closer to understanding the role of the actin monomer binding protein twinfilin in cytoskeletal dynamics. Our results support a model where twinfilin's function would be to deliver "inactive" ADP-G-actin to the sites of rapid actin polymerization in cells through direct interaction with capping protein.

INTRODUCTION

1. Actin

Actin is an extremely conserved protein that exists in all eukaryotic cells. In the majority it is the most abundant protein, and by forming different structures (figure 1) it participates in a great variety of cellular events. Mammalian actin forms more complex structures compared to the simple actin cytoskeleton in yeast (figure 1), and these mediate events such as cell movement, cell division, and endo- and exocytosis (figure 1). Higher eukaryotes have several isoforms of actin, coded for by a family of actin genes, while lower eukaryotes such as yeast have only one actin gene (reviewed in Sheterline et al., 1998). Nonetheless, the sequence homology between mammalian and yeast actin is almost 90 %.

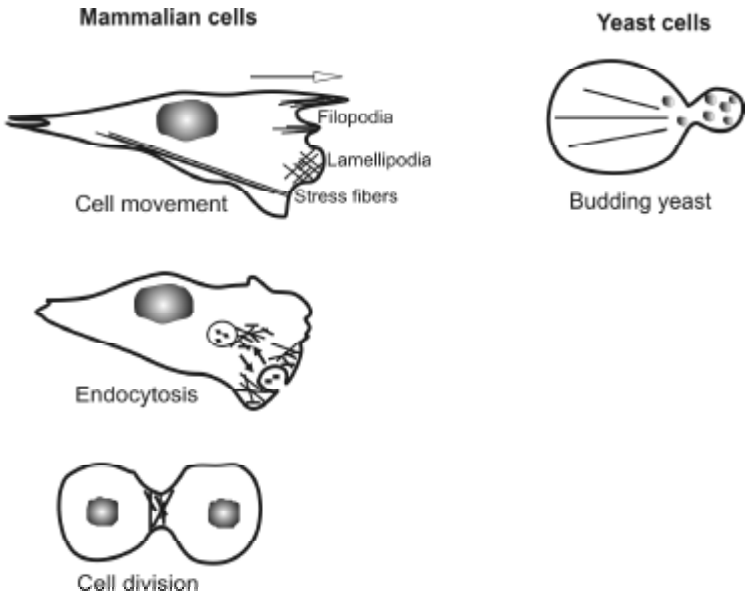

Figure 1. Schematic illustration of actin structures and actin-dependent cellular processes in mammalian and yeast cells. The filamentous actin structures in mammalian non-muscle cells include filopodia, lamellipodia, and stress fibres. These dynamic actin structures participate in a large variety of functions, exemplified here by movement, endocytosis, and cell division. In comparison, budding yeast has a simple actin cytoskeleton, which consists primarily of two morphologically different actin structures: actin patches and actin cables.

Cellular actin exists in two forms: as monomeric actin (G-actin) and as filamentous actin (F-actin) (figure 2). Every actin monomer has a nucleotide (ATP or ADP) and a divalent cation bound in a cleft formed between the lobes of the protein, and the conformation

and assembly kinetics of the monomer is modulated according to these bound cofactors. Linking monomers to each other, forming long "thread-like" structures, creates filamentous actin (figure 2). These actin filaments are polar structures with a fast growing barbed-end and a slow growing pointed-end, so named after the arrowhead pattern created when filaments are decorated with myosin (reviewed in Sheterline et al., 1998). The barbed end is favored for polymerization and monomers are preferentially added to this end in their ATP form (Wegner, 1976). After incorporation into the filament ATP is hydrolyzed to ADP and as such the monomer becomes less stable in the filament, leading in turn to depolymerization at the pointed end. This process is called treadmilling (figure 3), and takes place spontaneously in the test tube under physiological ionic conditions. The process can be several hundred times faster in living cells, however, due to the large number of actin binding proteins present (reviewed in Carlier, 1998).

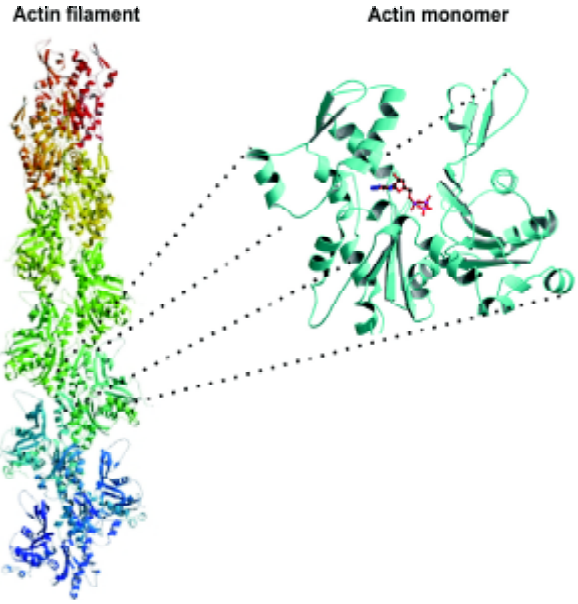

Figure 2. Actin monomers polymerize into filamentous actin. In cells actin exists in both monomeric (G-actin) and filamentous (F-actin) forms. A cleft between the two lobes of the actin monomer contains a nucleotide (ATP or ADP) as well as a divalent cation. By binding to each other, actin monomers form a polar filamentous structure.

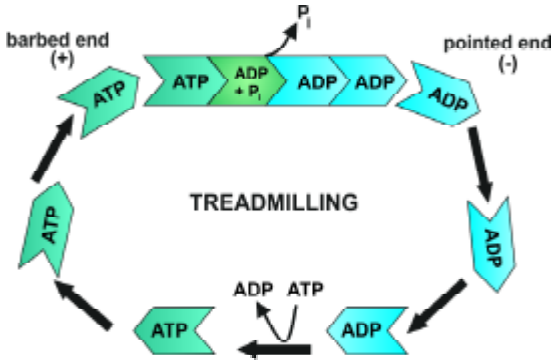

Figure 3. Actin filaments undergo treadmilling. Actin filaments are polar structures that favour incorporation of ATP-G-actin at their barbed end, and depolymerization of ADP-G-actin at their pointed end. The ATP is hydrolyzed after incorporation of an ATP-actin monomer to the filament, resulting in a less stable ADP-G-actin filament. Before a new round of polymerisation, the actin monomer, dissociated from the filament pointed-end, has to undergo nucleotide exchange to generate ATP-G-actin.

In muscle cells actin filaments are very stable, and together with myosin they form the sarcomere structure responsible for muscle contraction. On the contrary, actin filaments in non-muscle cells are very dynamic, responding to cellular signals in a strictly controlled manner. There are a vast number of proteins that interact with actin and modulate actin dynamics according to the needs of the cell. Filament bundling and cross-linking proteins (α-actinin, filamin etc.) help arrange actin filaments into higher order structures, while motor proteins (myosins) transport cargo along actin filaments. A large number of proteins are involved in the strict regulation of the coordinated growth and shortening of individual filaments according to the cells needs in response to cellular signals. Some of these proteins bind only filamentous or monomeric actin, while others bind both (reviewed in Sheterline et al., 1998).

2. Proteins regulating filament assembly

The importance of controlled actin polymerization is emphasized by the great variety of actin binding proteins fine-tuned to carry out this task, enabling the cell to function in a controlled manner. Actin filaments need to be polymerized and depolymerized, as well as organized into higher order structures, at a given location and in response to the correct cellular cues. Polymerization of actin filaments is a reaction that requires free actin filament ends as well as polymerization competent monomeric actin.

Since the critical concentration (0.1 mM) for polymerization of ATP-monomers is usually exceeded in the cytoplasm, cells need ways to hinder spontaneous polymerization and maintain a large pool of free monomers. This is accomplished both by actin monomer-binding proteins that regulate the availability of monomers for polymerization, as well as by proteins that bind filamentous actin and prevent subunit addition by capping filament ends. Both capping of filaments and interaction with different actin monomer binding proteins is required; alone neither would be sufficient to maintain a large enough pool of free actin monomers. Ways by which free filament ends needed for elongation can be created are uncapping of capped filaments, severing of old filaments, or nucleation of new filament ends (reviewed in Wear et al., 2000 and Condeelis, 2001).

Presented below are central proteins from each protein category that contribute to regulated filament assembly. Only the actin monomer-binding proteins that have been conserved through evolution, and thus play a universal role in regulating the actin monomer pool in cells, are described. Capping protein is presented as the most prominent filament capper in cells, and the Arp2/3 complex and formins are introduced as nucleators of new filaments.

2.1 Actin monomer binding proteins

There are six classes of actin monomer binding proteins that are evolutionarily conserved from yeast to mammals. These include the WASP protein family, verprolin/WIP and Srv2/CAP that are involved in signaling to the actin cytoskeleton, as well as ADF/cofilin, profilin, and twinfilin, which are directly involved in regulation of the actin monomer pool. β-thymosins, traditionally described as one main class of actin monomer binding proteins found in vertebrate cells, are not found in yeasts and invertebrates (reviewed in Pollard et al., 2000). Therefore, this class of actin monomer binding proteins will not be discussed here. The WASP family proteins, verprolin/WIP, and Svr2/CAP are all large proteins consisting of several different protein domains (figure 4). This enables them to interact with different signaling molecules and cytoskeletal proteins, as well as to bind actin monomers, allowing them to respond to varying cues and remodel the cytoskeleton accordingly. The smaller proteins ADF/cofilin, profilin, and twinfilin are more intimately involved in the actin filament treadmilling process, directly regulating the actin monomer pool.

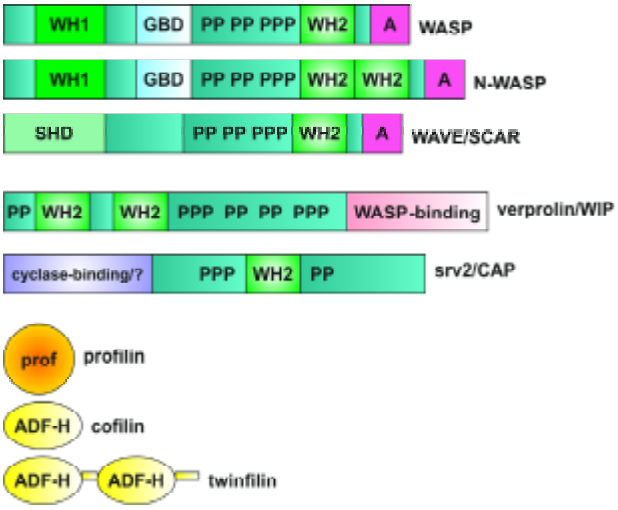

Figure 4. Actin binding domains are shared amongst actin monomer binding proteins. There are six functionally distinct classes of evolutionary conserved actin monomer binding proteins. The WASP-family proteins (WASP, N-WASP, and WAVE/SCAR), verprolin/WIP, and Srv2/CAP are multifunctional proteins consisting of several domains. Profilin, ADF/cofilin, and twinfilin are smaller proteins more directly involved in regulation of the actin monomer pool. These proteins bind actin through conserved actin binding domains. The large multidomain proteins (WASP-family proteins, verprolin/WIP and Srv2/CAP) all contain a WH2 domain(s), while ADF/cofilin and twinfilin contain ADF-H domain(s). Domain abbreviations: WH = WASP homology domain, GBD = GTPase binding domain, PP = proline rich domain, A = acidic domain, ADF-H = actin depolymerizing factor homology domain.

Nature has employed a sparse number of actin-binding motifs (WH2 domain, ADF-H domain, gelsolin domain and CH domain) that are used by the majority of actin binding proteins. The WH2 (WASP Homology 2) domain that mediates actin monomer interaction is found in three classes of ubiquitous actin binding proteins: the WASP-family of proteins, Srv2/CAP, and verprolin/WIP (reviewed in Paunola et al., 2002; see also figure 4). The ADF-H domain (Actin Depolymerizing Factor Homology), present in one copy in ADF/cofilin and in two copies in twinfilin (Lappalainen et al., 1998; figure 4), can interact with actin filaments and/or actin monomers. The gelsolin domain can bind both monomeric and filamentous actin, while the CH (Calponin Homology) domain appears to interact only with actin filaments (Puius et al., 1998).

2.1.1 WASP-family of proteins

The WASP (Wiskott-Aldrich Syndrome protein) family proteins are classified into two structurally distinct groups, WASPs (Derry et al., 1994), and WAVEs (WASP verprolin homologous protein; Miki et al., 1998) or SCARs (Suppressor of cAMP Receptor mutation; Bear et al., 1998). In mammals the family includes WASP (specific for blood platelets and leukocytes), N-WASP (neural WASP, which is actually ubiquitous; Miki et al., 1996), and at least four WAVE/SCAR proteins. In budding yeast there is only one WASP/SCAR homologue, Bee1/Las17 (Li 1997; Madania et al., 1999).

The proteins in this family are composed of several domains (figure 4) and they interact with a large number of proteins, acting as intermediators between signaling molecules and the actin cytoskeleton. The carboxy-terminal regions of these proteins are very similar to each other, but their variable amino-termini enable them to respond to different signaling molecules. In their C-termini these proteins have an acidic domain (A) that binds and activates the actin filament nucleator Arp2/3. The WH2 domain, present in one or two copies, mediates binding to actin monomers. The proline rich domain (P) enables binding to profilin and SH3 domain containing proteins. WASP and N-WASP have a N-terminal GBD (GTPase Binding Domain) domain consisting of a CRIB (Cdc42/Rac Interactive Binding) motif that binds the small GTPase Cdc42 (see chapter 3.3.1). WAVE/SCAR proteins lack this domain and thus seems to be differentially regulated (reviewed in Mullins, 2000). Yeast Las17 resembles WASP proteins in its overall domain organization, but it also lacks a GTPase binding motif and does not interact directly with Cdc42 (Lechler et al, 2001), being in this aspect more like WAVE/SCAR proteins.

The regulatory mechanisms of WASP and WAVE/SCAR proteins differ greatly, although small GTPases are involved in activation of both protein classes. While Cdc42 acts through WASP and N-WASP, Rac acts through WAVE/SCAR (Miki et al., 1998). WASP family proteins are normally autoinhibited by their WA (A + WH2 domains) domain being bound to their GBD domain. Once this autoinhibition is released by bind-

ing of WASP ligands Cdc42, PIP_2, or SH3 containing proteins, WASPs can function as an activator of the Arp2/3 complex (Rothagi et al., 2000; Higgs and Pollard, 2000; Prehoda et al., 2000).

The lack of a GBD domain in WAVE/SCAR proteins indicates a different regulatory mechanism without direct GTPase binding. Recombinant WAVE1 is constitutively active. However, in cells WAVE1 is found in an inactive multiprotein complex. Dissociation of this complex by Rac1 releases active WAVE1 and leads to Arp2/3-mediated actin filament nucleation (Eden et al., 2002).

The regulation of yeast Las17, which also lacks a GBD domain, resembles that of WAVE/SCAR proteins even though its domain organization is more similar to WASP proteins. Las17 is a constitutively active protein with multiple ligands, such as Sla1 and Bbc1, negatively regulating its activity (Rodal et al., 2003).

2.1.2 Verprolin/WIP

Originally identified from a yeast two-hybrid screen as a WASP interacting protein (Ramesh et al., 1997), WIP (WASP Interacting Protein) has several binding partners but its precise mechanism of influencing cytoskeletal dynamics remains unclear. It binds WASP/N-WASP through its C-terminal domain (Volkman et al., 2002), has a central proline rich region, and interacts with actin through its N-terminal WH2 domains (Ramesh et al., 1997; Martinez-Quiles et al., 2001; see also figure 4). Similarly to WIP, the yeast homologue End5/verprolin also interacts with Bee1/Las17 (N-WASP) (Vaduva et al., 1997).

End5/verprolin and Bee1/Las17 are both components of the cortical actin patches and are required for actin cytoskeleton organization and endocytosis (Li, 1997; Vaduva et al., 1997; Naqvi et al., 1998). Yeast End5/verprolin contributes to induction of actin polymerization also by interacting with other actin patch components: myosin 1 proteins Myo3 and Myo5 (Evangelista et al., 2000).

Similarly to yeast End5/verprolin, mammalian WIP also influences actin polymerization. Through interaction with its different binding partners, WIP can influence actin polymerization by Arp2/3 in alternative ways. WIP has no GBD of its own and thus is unlikely to interact directly with Cdc42, but in complex with N-WASP it can retard actin polymerization mediated by Cdc42/N-WASP-activated Arp2/3 *in vitro* (Martinez-Quiles et al., 2001). In contrast, WIP can activate Arp2/3 mediated polymerization when bound to the Arp2/3-activator cortactin (Kinley et al., 2003). Since WIP has been shown to bind and stabilize actin filaments in addition to binding monomeric actin (Martinez-Quiles et al., 2001), the WIP-cortactin complex may influence Arp2/3-mediated actin net-

works by activating polymerization and inhibiting depolymerization and debranching (Kinley et al., 2003). WIP has also been shown to interact with additional proteins involved in cytoskeletal dynamics, including profilin (Ramesh et al., 1997) and the adapter protein Nck (Anton et al., 1998). During actin polymerization induced by the *Vaccinia* virus, recruitment of WIP is required for correct localization of N-WASP, and consequently the actin based motility of *Vaccinia* (Moreau et al., 2000).

2.1.3 Srv2/CAP

CAP (Cyclase-Associated Protein) is a highly conserved protein found in a wide range of organisms including yeast, plants, insects, and mammals (reviewed in Hubberstey and Mottillo, 2002). Srv2/CAP was first identified in *Saccharomyces cerevisiae* as a protein involved in the Ras signaling pathway (Fedor-Chaiken et al., 1990; Field et al., 1990). However, the Ras dependent activation of adenylyl cyclase appears to be separate from the crucial actin organization function of Srv2/CAP, and seems relevant only in fungi (Vojtek et al., 1991). At least two CAP homologues, CAP1 and CAP2, which are 64 % identical at the amino acid level, exist in mammals (Yu et al., 1994).

CAP is composed of four structural domains: a central proline rich domain, C- and N-terminal domains, as well as a domain responsible for oligomerization (reviewed in Hubberstey and Motillo, 2002; see also figure 4). The highly conserved C-terminal domain has been shown to bind G-actin in all CAPs tested (Kawamukai et al., 1992; Freeman et al., 1995). The C-terminal region of Srv2/CAP also contains a WH2 domain, but its role in actin binding remains unknown. The central proline-rich domain of Srv2/CAP contains a recognition sequence for SH3 proteins and has been shown to bind Abp1 in yeast (Freeman et al., 1995; Balcer et al., 2003). This domain could also be a potential binding site for profilin. Although no single multimerization domain of CAP has been defined, a region in the N-terminus is known to be important for multimerization, and mutagenesis studies have shown that localization and multimerization may be linked (Yu et al., 1999).

In yeast, loss of function of the C-terminal domain of Srv2/CAP can be compensated for by overexpression of the actin binding protein profilin, indicating overlapping tasks of these proteins (Vojtek et al., 1991). CAP was originally thought to contribute to actin dynamics as an actin monomer sequestering protein (reviewed in Hubberstey and Motillo, 2002). Recent studies, however, demonstrate that the function of CAP is to enhance actin filament turnover by recycling actin monomers and ADF/cofilin for new rounds of actin filament polymerization and depolymerization, respectively (Moriyama and Yahara, 2002; Balcer et al., 2003; Bertling et al., 2004). At least mammalian CAPs interact directly with the cofilin-actin monomer complex through their conserved N-terminal domain (Moriyama and Yahara, 2002).

2.1.4 Profilin

Profilin is a small (Mw ~ 12-16 kD) ubiquitous protein found in all eukaryotic organsims. It was the first actin monomer binding protein to be discovered (Carlsson et al. 1977) and it plays a complex role in actin dynamics. In vertebrates there are at least four functionally distinct profilin isoforms: profilin I, two splice variants of profilin II (Di Nardo et al., 2000; Lambrechts et al., 2000), and profilin III (Hu et al., 2001). Profilin I is the most widely occuring isoform, and seems to be present in all vertebrate cells (Witke et al., 2001).

Profilin sequesters actin monomers (Carlsson et al., 1977), catalyzes nucleotide exchange on the actin monomer from ADP to ATP (Mockrin and Korn, 1980; Goldschmidt-Clermont et al., 1991), and promotes barbed end assembly of actin filaments (Pantaloni and Carlier, 1993). In addition to binding actin monomers, profilin interacts with phosphoinositides (Machesky and Poland, 1993; Skare and Karlsson, 2002) as well as with a number of proteins containing poly-proline rich sequences, such as verprolin/WIP (Ramesh et al., 1997), VASP-family proteins (reviewed in Krause et al., 2003), and yeast formins Bni1p and Bnr1p (Evangelista et al., 1997; Imamura et al., 1997). Profilin may thus function as a link between certain signaling pathways and the actin cytoskeleton.

In all organisms studied, profilin is shown to be crucial for creation and maintenance of a polarized actin cytoskeleton (reviewed in Ayscough, 1998). This is well demonstrated in yeast cells lacking profilin (Pfy1p), since they grow extremely slowly and have abnormal morphology and actin cytoskeletons (Haarer et al., 1990). The biological significance of profilin´s ability to enhance nucleotide exchange on actin has been debated due to the varying degree in which this activity occurs in different organisms. In humans, profilin increases nucleotide exchange 40 - 1000 fold compared to actin alone (Perelroizen et al., 1996), in *S. cerevisiae* only three-fold (Eads et al., 1998), while in plants profilin shows no nucleotide catalyzing activity (Perelroizen et al., 1996). Extensive mutagenesis studies in yeast underline the importance of profilin's ability to exchange the nucleotide on the actin monomer and suggests that nucleotide exchange could be the rate limiting step in actin polymerization (Wolven et al., 2000).

In addition to localizing to the sites of rapid actin dynamics in cells, profilin is also found to occur in the cell nucleus. The localization of profilin to Cajal bodies, conserved nuclear compartments involved in RNP maturation (reviewed in Ogg and Lamond, 2001), suggests that it also carries out other cellular roles besides participation in actin filament turnover. It is possible that this nuclear profilin, or perhaps profilin-actin, is involved in pre-mRNA processing (Skare et al., 2003).

2.1.5 ADF/Cofilin

ADF/cofilins are small (Mw ~ 15-20 kDa) actin binding proteins that are composed entirely of one ADF-H-domain (figure 4). They are evolutionarily conserved and required for yeasts, worm and flie viability (Moon et al., 1993; McKim et al., 1994; Gunsalus et al., 1995). Plants and vertebrates have several ADF/cofilin genes that have different expression patterns and biochemical properties. Many other eukaryotes, including yeast, have only one ADF/cofilin gene (reviewed in Maciver and Hussey, 2002). ADF/cofilin differs from the other actin monomer binding proteins in that it binds both actin filaments as well as actin monomers. It prefers ADP-actin to ATP-actin (Maciver and Weeds, 1994) and contributes to cytoskeletal dynamics by increasing depolymerization of actin monomers from the pointed end of the actin filaments (Carlier et al., 1997). Upon binding to actin filaments, ADF/cofilin also induces a twist in the filament. This structural change is believed to be the mechanism of ADF/cofilin´s actin depolymerization/severing activity (McGough et al., 1997)

The activity of ADF/cofilin can be regulated by numerous mechanisms including pH (Bernstein et al., 2000; Yonezawa et al., 1985), phosphorylation (Morgan et al., 1993; Agnew et al., 1995), $PI(4,5)P_2$ (Yonezawa et al., 1990), and by interactions with other actin binding proteins. Actin depolymerization by ADF/cofilin has been shown to be pH dependent *in vitro* and requires an alkaline environment with a pH above 7.2 (Yonezawa et al., 1985). ADF/cofilin also binds $PI(4,5)P_2$, and at least *in vitro* its activity can be regulated by phospholipids. The mechanism of $PI(4,5)P_2$ inhibition is probably due to overlapping binding sites between actin and $PI(4,5)P_2$ binding on the surface of ADF/cofilin (Yonezawa et al., 1990 & 1991; Ojala et al., 2001). The best characterized regulatory mechanism of ADF/cofilin is phosphorylation by LIM kinase in mammalian cells, which occurs through a signal transduction cascade where upstream kinases (Pak1, ROCK, protein kinase C) phosphorylate LIM kinase upon activation by small G-proteins or diaglycerol (DAG). LIM kinase catalyzes phosphorylation of ADF/cofilin rendering it unable to bind actin (Arber et al., 1998; Yang et al., 1998). In contrast to vertebrates, yeast ADF/cofilin appears not to be regulated by phosphorylation (Lappalainen and Drubin, 1997).

The presence of other actin binding proteins also influences ADF/cofilin's ability to depolymerize and sever actin filaments. ADF/cofilin competes for actin binding with profilin (Blanchoin and Pollard, 1998) and with tropomyosins (reviewed in Bamburg, 1999). Furthermore, ADF/cofilin also interacts with an evolutionarily conserved protein, Aip1. This interaction stimulates the actin filament depolymerization activity of ADF/cofilin, perhaps through formation of a ternary complex of actin-ADF/cofilin-Aip1 (Rodal et al., 1999).

2.1.6 Twinfilin

Twinfilin is a ubiquitous actin monomer binding protein, first identified from budding yeast (Goode et al 1998). It is found in organisms from yeast to mammals, but has not been identified in plants. While lower eukaryotes such as yeast have only one twinfilin, there are two differentially expressed isoforms of twinfilin in mice (Vartianen et al. 2003).

Twinfilin is composed of two ADF-homology (Actin Depolymerization Factor) domains (figure 4) resembling that of ADF/cofilin, hence its name "twinfilin". The ADF-H domains of twinfilin are 20 % identical to each other and to ADF/cofilin, but unlike ADF/cofilin, twinfilin binds solely to actin monomers and does not interact with actin filaments. Accordingly, the residues important for actin monomer binding in ADF/cofilin are well conserved in twinfilin, while the residues important for filament binding are not (Lappalainen et al., 1998).

Twinfilin binds actin monomers with a 1:1 stoichiometry and prevents nucleotide exchange on the monomer upon binding (Goode et al. 1998, Vartiainen et al., 2000). The phenotypes seen in yeast upon over-expression or deletion of twinfilin are indicative of twinfilin's involvement in regulation of actin dynamics. Over-expressing twinfilin in yeast leads to enlarged cortical actin patches while deletion leads to a disturbed budding pattern and also results in synthetic lethality with certain yeast *ADF/cofilin* and *profilin* mutants (Goode et al. 1998). The importance of twinfilin for actin-dependent developmental processes in multicellular organisms is demonstrated by defects, such as aberrant bristle and eye morphology, in twinfilin mutant *Drosophila* (Wahlström et al. 2001).

The cellular localization of twinfilin also speaks for its involvement in actin dynamics. Mouse twinfilin, which is 25% identical to the yeast protein at the amino acid level, shows diffuse cytoplasmic localization, but additionally localizes to cortical actin structures rich in monomeric actin (Vartiainen et al. 2000). In developing *Drosophila* bristles twinfilin shows diffuse cytoplasmic staining, but it also localizes to the ends of actin filament bundles (Wahlström et al., 2001).

2.2 Proteins binding filamentous actin

As mentioned above, actin filament binding proteins can be divided into motor proteins, proteins arranging filaments into higher order structures, and proteins influencing cytoskeletal dynamics at the single filament level. Only three ubiquitous proteins from the last category will be discussed further here: capping protein, which caps the barbed ends of filaments, and Arp2/3 and formins, which nucleate *de novo* formation of actin filaments.

2.2.1 Capping protein

Capping protein is a heterodimeric protein consisting of α and β subunits, with molecular weights of 31-36 kD and 28-32 kD (Pollard and Cooper, 1986), respectively. Capping protein is found in a wide variety of organisms and tissues where it binds to the barbed-end of actin filaments with high affinity (K_d ~ 1 nM) and 1:1 stoichiometry (Caldwell et al., 1989; Schafer et al., 1996), thereby blocking the addition and loss of actin monomers. In muscle cells, capping protein (CapZ) is found at the Z lines (Casella et al., 1987), while non-muscle capping protein is mainly localized at the cortical actin cytoskeleton (Amatruda and Cooper, 1992; Schafer et al., 1992). In *S. cerevisiae*, capping protein co-localizes with actin to the cortical actin patches but is absent from cytoplasmic actin cables (Amatruda and Cooper, 1992).

The crystal structure of chicken sarcomeric capping protein indicates that it binds actin filament ends with two mobile extensions (Yamashita et al., 2003). Mutagenesis studies confirm binding occurs by two independent "tentacles" capping the barbed end of the filament (Wear et al., 2003). Capping is proposed to occur by simultaneous binding of several actin subunits at the barbed-end, since capping protein mutants with either tentacle can cap filament ends (Wear et al., 2003).

Capping protein is one of the key regulators of cytoskeletal dynamics, crucial for rapid elongation in response to cell signaling. Directed movement requires systematic actin remodeling, and by blocking a large number of actin filament ends capping protein directs elongation to the few uncapped filaments, resulting in faster growth of these specific filaments (Pantaloni et al., 2001). The off-rate of capping protein from actin filament barbed ends is too slow (half-life of ~ 30 min) for creation of free barbed ends by spontaneous dissociation. Uncapping has to be enhanced in a controlled fashion and phosphoinositides, especially $PI(4,5)P_2$, have been shown to be second messengers regulating the activity of capping protein (Amatruda and Cooper, 1992). The mechanism for this inhibition seems to be rapid dissociation of capping protein from the filament barbed end upon $PI(4,5)P_2$ binding, thus allowing controlled elongation (Schafer et al., 1996). The crystal structure of capping protein also supports this view, since the plausible $PI(4,5)P_2$ binding sites seem to reside near capping protein´s actin binding interface (Yamashita et al., 2003).

2.2.2 Arp2/3

The Arp2/3 complex consists of seven proteins, including actin-related proteins Arp2 and Arp3. In all eukaryotes the Arp2/3 complex is a multifunctional organizer of actin filaments (Machesky et al., 1994; Machesky and Gould, 1999). It can initiate polymerization of new filaments as well as cap and cross-link filaments. The exact mechanism by

which Arp2/3 nucleates polymerization remains unclear, but it has been proposed that a conformational change within this protein complex brings Arp2 and Arp3 close to each other, mimicking a filament barbed-end and allowing polymerization (Robinson et al., 2001).

In yeast the Arp2/3 complex is an essential component of the cortical actin patches (Winter et al., 1997). Both Arp2 and Arp3 have been shown to be involved in the maintenance of the correct organization of the actin patches, while Arp3 is required also for the movement of actin patches *in vivo* (Winter et al., 1997; Moreau et al., 1996).

The Arp2/3 complex shows little activity on its own, but is activated by a class of proteins called nucleation promoting factors (NPFs). These contain a region necessary for binding Arp2/3, but in order to activate the complex they need an adjacent actin binding site that, depending on the protein, mediates binding to either G- or F-actin. NPFs with the WH2-domain G-actin binding motif include WASP, N-WASP, WAVE, and Las17p/Bee1p. These activate the Arp2/3 complex by forming a ternary complex of Arp2/3-NPF-G-actin, but full activation additionally requires interaction with a pre-existing "mother filament" (Machesky et al., 1999). Two different models have been proposed for Arp2/3 function, and they both share the idea of nucleation and filament network formation taking place simultaneously. The "dendritic model" (Pollard et al., 2000) is based on Arp2/3 binding to the sides and pointed end of actin filaments (Mullins et al., 1998), and describes Arp2/3 complex-NPF nucleation of daughter filaments occurring from the side of the mother filament. In the "barbed end branching model" proposed by Pantaloni et al. (2000) the Arp2/3 complex-NPF assembly co-polymerizes with actin at the barbed end of the filament, branching the filament as it grows. Recent evidence favors the dendritic model although it seems that the ATP-actin preference of the Arp2/3 complex directs branching towards the barbed end of the filament (reviewed in Welch and Mullins, 2002).

2.2.3 Formins

Formins are conserved from yeast to mammals. They are multi-domain proteins defined by a conserved formin-homology 2 (FH2) domain that is required to nucleate actin filaments (Pruyne et al., 2002). A GTPase binding domain (GBD) relevant for protein regulation is present in the majority of formins. Most formins also have a proline rich FH1 domain, which binds profilin and some other proteins. A third FH domain, FH3, present in some formins, is involved in its subcellular localization (Wallar and Alberts, 2003).

Formins nucleate filaments by actin dimer stabilization. The FH2 domain is sufficient for nucleation, but the FH1 domain increases the efficiency by interacting with profilin-

actin. Formins are also described as “leaky cappers”, which means that they allow some polymerization and depolymerization (~ 50% of normal efficiency) to occur while bound to the actin filament barbed end (Pring et al., 2003). By binding to the filament barbed end and allowing some polymerization to occur, formins protect the barbed end from tight cappers (such as capping protein) and promote filament elongation. The “leaky cap” travels with the actin filament barbed end as it grows, and is probably formed by an oligomer of at least two formins (Zigmond et al., 2003).

The Arp2/3 complex, presented above, nucleates branched filaments. Formins, on the other hand, generate unbranched filaments that are anchored at their barbed end. The apparent benefit of branched filaments is that they create a dendritic network optimized for protrusive force, while the stability of straight filaments is beneficial for contraction, which arises from movement of myosin along the filaments. The different nucleators may thus have arisen to generate actin filaments with different mechanical functions (reviewed in Zigmond, 2003). In mammalian cells Rac and Cdc42 activate Arp2/3, through interaction with WASP family proteins, to drive protrusion of filopodia and lamellipodia. Rho activates formins and myosin for contraction and transport, and is required for stress fiber formation (Watanabe et al., 1999). In accordance with this, the Arp2/3 complex in budding yeast is crucial for actin assembly in the cortical actin patches. However, it is not required for actin cable formation. This function is instead regulated by the formins Bni1p and Bnr1 (Evangelista et al., 2002). Bni1p nucleates the actin cables in yeast and anchors their barbed ends to the bud tip. These actin cables are then used for transport of vesicles towards the bud by myosin motor proteins and for nuclear positioning and spindle orientation (Pruyne and Bretcher, 2000 b).

Diaphanous-related formins, such as yeast Bni1 and mammalian mDia1, are regulated by an autoinhibitory mechanism, where they are kept inactive by an association between GBD and part of the FH2 domain (Watanabe et al., 1999; Alberts et al., 2001; Li and Higgs, 2003) By a mechanism similar to that of WASp protein activation by GTP-Cdc42, binding of GTP-Rho to the GBD domain relieves this inhibition and releases the C-terminus, containing the FH1, FH2, and COOH domains that stimulate actin assembly (Rohatgi et al., 1999).

3. The actin cytoskeleton of yeast

Saccharomyces cerevisiae, baker’s yeast, has been successfully used as a model organism to study numerous cellular functions. It is especially well suited for studying the actin cytoskeleton since it contains morphologically distinct actin structures resembling those of higher eukaryotes, and because it shows cell cycle dependent actin reorganization (figure 5).

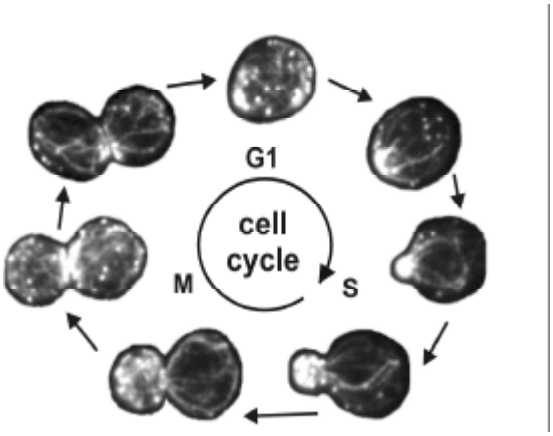

Figure 5. The actin cytoskeleton of *Saccharomyces cerevisiae* shows cell cycle-dependent polarization. During the cell cycle, the yeast actin cytoskeleton exhibits cell cycle dependent polarization, with the actin patches polarizing to the growing bud and the cables extending through the cytoplasm oriented towards the bud. Picture modified from (Karpova et al., 1998).

3.1 Yeast as a model organism

Of all the model organisms with which geneticist work, *S. cerevisiae* is probably the most thoroughly analyzed. Its genome sequence was published in 1996 (Goffeau et al., 1996), making it the first eukaryote whose entire DNA was known. With a small genome of about 6000 genes along with highly developed tools for their manipulation, yeast is an excellent model organism for genetic studies. Yeast is also cheap and fast to cultivate, as it grows with a doubling time of approximately 90 minutes.

As a model organism, the resemblance to higher eukaryotes is naturally of central importance. In spite of its microscopic size and simple appearance, yeast is remarkably similar to mammalian cells. For example yeast actin is 88% identical to rabbit muscle actin at the amino acid level and the proteins are also very similar biochemically, indicating that what is learned in yeast can be applied in a wider sense (Welch et al., 1994). Not only is actin conserved from yeast to mammals, but most actin interacting proteins and their regulators are also evolutionarily conserved.

In contrast to higher eukaryotes that often posses several protein isoforms, which complicate functional analysis of a protein, yeast is simple and usually makes do with only one form of most proteins. With only one actin (*ACT1*), as well as only one capping protein (*CAP1* and *CAP2* encoding for α and β subunits, respectively), one ADF/cofilin (*COF1*), and one twinfilin (*TWF1*), yeast offers a great opportunity for studying

the phenotypes of mutants lacking a specific protein, thus revealing information conserning its function.

3.2 Actin structures of *Saccharomyces cerevisiae*

The actin cytoskeleton of yeast is very simple and is usually described to consist only of two morphologically distinct structures, actin cables and actin patches (figure 1 and 5). In addition to these two main structures, the yeast actin cytoskeleton also includes the cytokinetic ring that mediates cell division. Yeast also have a structure referred to as the cap, which consists of regulatory and cytoskeletal proteins and establishes polarity of actin cables and patches (reviewed in Pruyne and Bretcher, 2000b). Several viable yeast mutants lacking actin cables have been described, but only one, *bee1/las1*, has been described as lacking actin patches (Li, 1997). The function of the actin patches and cables have long remained unclear at the molecular level, but recent studies have brought new understanding to these cytoskeletal structures.

3.2.1 Actin patches

Although the cortical actin patches were already discovered two decades ago (Adams and Pringle, 1984) their exact biochemical composition and precise function has long remained unclear. Cortical actin patches are biochemically complex and share many of the same components as cortical structures in vertebrates, including capping protein and Arp2/3. These structures differ, however, in the sense that they are required for motility in vertebrates but not in yeast (Karpova et al., 1998). Different subpopulations of actin patches have been thought to exist since varying results regarding protein composition of the patches have been obtained. These observations have recently been explained by different proteins being localized in the patch at different times, resulting in patch intermediates of variable protein compositions (Kaksonen et al., 2003).

The actin patches in yeast are highly motile structures that can move long distances in the cell at great speed (Waddle et al., 1996). During the cell cycle they show a characteristic asymmetric distribution and concentrate at the marked bud site where bud emerge begins (figure 5). Actin patches are found exclusively in the bud once it matures (Adams and Pringle, 1984). They are essential for normal growth, during which they are located near the sites for exocytosis (Adams and Pringle, 1984). They have also been suggested to participate in endocytosis **(**Engqvist-Goldstein and Drubin, 2003). To assemble and participate in endocytosis the cortical actin patches require Arp2/3 (Winter et al., 1999).

Kaksonen et al. (2003) have recently addressed some of the confusing issues concerning the cortical actin patches and presented a pathway for their endocytic internalization.

Their model describes step-wise formation and subsequent internalization of an actin patch, beginning with early actin patch components such as Arp2/3 activators (Las17p) and scaffold proteins assembling in a nonmotile complex at the plasma membrane. Only later are actin, Abp1, and Arp2/3 recruited to the patch, which in turn results in slow actin-based movement inwards from the plasma membrane and dissociation of the early components from the patch. This pathway fits well with previously obtained data, explaining the existence of patches of different protein composition (Warren et al., 2002) as well as claims both for and against patch movement being actin dependent (Lappalainen and Drubin, 1997; Winter et al., 1997). The different velocity patch movements are also suggested to occur through different mechanisms, so that the slow internalization step motility is actin polymerization dependent while the fast movement of the internalized patch does not depend on actin dynamics (Kaksonen et al., 2003).

3.2.2 Actin cables

The actin cables are linear structures composed of bundles of actin filaments that run through the cytoplasm (Karpova et al., 1998), always oriented towards the cap (figure 5). Unlike in actin patches, where nucleation of actin filaments occurs by the Arp2/3 complex, the linear actin cables are stimulated by the formins Bni1p and Bnr1p (Bni1p related protein1) independently of Arp2/3 (Evangelista et al., 2002). Loss of both Bni1p and Bnr1p in yeast is lethal (Vallen et al., 2000). Bni1p binds barbed ends of filaments and allows growth while bound, making it a unique actin filament nucleator (Pruyne et al., 2002). This novel nucleator could function in similar ways in other eukaryotes, providing an explanation for how the various actin structures in cells are accomplished. In mammalian cells the long linear stress fibers could potentially be candidates for nucleation by formins (Watanabe et al., 1999).

Secretory vesicles are transported along the actin cables, allowing polarized growth and organelle segregation (Pruyne and Bretscher, 2000a). Unlike animal cells that need microtubules for long-range transport, actin-dependent transport seems sufficient in the small yeast cells. The unconventional myosin V proteins, Myo2 and Myo4, function as transporters along the actin cables, moving towards the barbed end of actin filaments. The cargo they carry differs, Myo2 transports protein complexes and membrane bound compartments, while Myo4 transports mRNAs (reviewed in Bretscher, 2003). Spindle orientation in yeast also requires transport of microtubule ends along actin cables (Hwang et al., 2003).

3.3 Regulation of the cytoskeleton

Regulation of the actin cytoskeleton in response to cellular cues is a complex event involving numerous proteins and signaling molecules that allow the cell to respond prop-

erly to different signals. In almost all motile eukaryotic cells, directed motility requires polymerization of actin filaments assembled into a network at the leading edge of the cell driving membrane protrusion. Cellular signaling pathways must somehow control this process, allowing the cell to move in its desired direction and to change direction in response to extracellular cues. One such a pathway involves the Rho family of GTPases that mediate external signals received by receptors at the plasma membrane to WASP/Scar proteins. Downstream from WASP/Scar the Arp2/3 complex induces *de novo* nucleation and cross-linking of actin filaments, thus influencing the actin cytoskeleton (Machesky and Gould, 1999).

Even though yeast is not a motile organism it is well suited for studying signaling to the actin cytoskeleton. Yeast cells exhibit cell cycle dependent reorganization of the actin cytoskeleton, and asymmetrical polarization of actin takes place also during formation of mating projections (Ayscough and Drubin, 1998). *S. cerevisiae* divides through budding and the budding pattern is determined based on both genotype as well as available nutrients (Drubin and Nelson, 1996). MATa/MATα diploid cells have a bipolar budding pattern where the daughter cell buds close to the previous budding site or opposite this site (Chant and Pringle, 1995). The actin cytoskeleton plays a central role in bipolar budding, as the cortical actin patches are concentrated at the bud region and actin cables are oriented along the long axis of the mother cell-bud (Drubin, 1991).

3.3.1 Rho family of small GTPases

Cdc42, RhoA, and Rac1 are the most widely investigated signaling molecules controlling the actin cytoskeleton. They belong to the family of small GTPases that cycle between an active GTP-form and an inactive GDP-form. The different activation states of the GTPases are regulated by specific regulatory factors: guanine nucleotide exchange factors (GEFs) promote GDP-GTP exchange, GTPase activating proteins (GAPs) stimulate inactivation, and Rho guanine nucleotide dissociation inhibitors (Rho-GDIs) stabilize the inactive protein form.

Mammalian Cdc42, Rho, and Rac influence the actin cytoskeleton through varying and complex signaling cascades, resulting in different actin structures. Cdc42 controls formation of filopodia, Rho induces formation of stress fibers, and Rac regulates formation of lamellipodia in mammalian cells. Many of the signaling pathways used by Cdc42, Rho, and Rac to transmit their signals to actin are largely overlapping. Some of these signaling pathways are characterized in great detail. Cdc42, Rho, and Rac can all, for example, regulate actin dynamics through activation of LIM kinase, which phosphorylates ADF/cofilin. Cdc42 also influences actin by signaling through N-WASP, which activates Arp2/3. Rho can influence actin assembly through activation of formin mDia, as

well as by activation of myosin, through MLCK (reviewed in Bishop and Hall, 2000).

Yeast has six Rho family proteins, Cdc42p and Rho1 to 5p. Four of these have been shown to interact with the formins Bni1 and Bnr1 that direct the assembly of actin cables. Normally Rho3p and Rho4p interact to activate Bni1 and Bnr1, while Rho1p is needed for activation at higher temperatures. Cdc42p is required for properly organized cable orientation during bud growth (Dong et al., 2003). It is thus central for guiding polarization of the actin cytoskeleton in yeast, and polarity can be modulated by regulating the localization and activation of Cdc42 (Pruyne and Bretscher, 2000b).

3.3.2 PI(4,5)P_2 in actin cytoskeleton regulation

The cleavage products of PI(4,5)P_2, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), are traditionally described as central signaling molecules in the cell. IP_3 influences several proteins by causing the cellular Ca^{2+} levels to rise, while the main function of DAG is activation of protein kinase C (PKC), which phosphorylates target proteins in the cell. For a long time it was thought that the only function of PI(4,5)P_2 was generation of IP_3 and DAG, but it has later become apparent that PI(4,5)P_2 itself is also an important regulatory molecule interacting directly with numerous proteins in the cell (Toker, 1998). Much is still unclear about the details of how PI(4,5)P_2 and other phospholipids function to regulate the large number of cytoskeletal proteins to which they bind (reportedly over 20) (Machesky and Insall, 1999).

Recently the focus of PI(4,5)P_2 regulation has turned towards phosphatidylinositol phosphate kinases (PIP kinases) (Reviewed in Doughman et al., 2003). There are two subfamilies of PIP kinases that produce PI(4,5)P_2 from different substrate pools by unique mechanisms. The classical way to produce PI(4,5)P_2 is phosphorylation of PI(4)P by type I PIP kinases (reviewed in Anderson et al., 1999), but PI(4,5)P_2 can also be produced by type II kinases using PI(5)P as a substrate (Rameh et al., 1997). As type II kinases are not found in yeast, and as they do not regulate actin cytoskeleton reorganization in mammalian cells, they are not further discussed in this context. The type I PIP kinase sub-family consist of several isoforms, and their splice variants have been shown to be differentially localized in the cell synthesizing PI(4,5)P_2 at distinct sites. Type I PIP kinase in yeast (Mss4p) localizes to the plasma membrane and is involved in actin reorganization, indicating that type I kinases are functionally conserved. The Rho family of small G-proteins (Rho, Rac, and Arf) regulate type I PIP kinase localization and activation and thereby influence targeted production of PI(4,5)P_2. PIP kinase activity is further regulated by phosphorylation, which inhibits these kinases in both yeast and mammalian cells (reviewed in Doughman et al., 2003).

PI(4,5)P_2 binds many actin binding proteins, regulating them in different ways (reviewed

in Hilpelä et al., 2004). It downregulates proteins (such as capping protein, ADF/cofilin, and profilin) that induce depolymerization, and activates proteins (such as the WASPs) that induce polymerization. $PI(4,5)P_2$ has been shown to promote actin filament assembly *in vivo* and *in vitro*, which is in good agreement with its effects on the actin binding proteins it interacts with. Actin polymerization can be guided to specific regions of the cell, such as the plasma membrane, by localization of $PI(4,5)P_2$ (reviewed in Hilpelä et al., 2004).

AIMS OF THE STUDY

This study was carried out to investigate the central properties of the actin binding protein twinfilin, aiming to understand the function and cell biological role of this protein. Twinfilin has only recently been identified as a protein with homology to ADF/cofilin (Lappalainen et al., 1998, Goode et al., 1998) and very little was known about it at the beginning of this study. Therefore, the goal of this work was to identify the role of twinfilin in actin dynamics.

Specific aims were:

- Investigation of the amount and localization of twinfilin in yeast cells.
- Identification of binding partners for twinfilin
- Characterization of the binding sites in twinfilin mediating interactions with its binding partners
- Investigation of the biological significance of twinfilin's protein interactions

MATERIALS AND METHODS

The experimental methods used in this thesis work are listed in table 1, and detailed descriptions are found in the original publications as indicated. Table 2 contains a list of the yeast strains used in this study.

Table 1. Methods used in this study

Method	***Publication***
Affinity purification of antibodies	I
Site-directed mutagenesis and plasmid construction	I, II, III
SDS-PAGE	I, II, III
Native gel electrophoresis assays	I, III
Protein expression and purification	I, II, III
Actin filament sedimentation assay	I
Actin depolymerization assay (pyrene-actin)	I
NBD-actin assay	II, III
Immunofluorescence microscopy	I, III
Western blotting	I, III
Co-immunoprecipitation	I, III
Yeast tetrad analysis	III
Yeast crosses	III

Table 2. Yeast strains used in this study

Strain	Genotype
BGY11	*MAT* α *his3 leu2 ura3 ade2 trp1 lys2 crn1D::LEU2*
DAY32	*MAT* **a** *leu2D1 ura3-52 trp1D63 his3D200 aip1D::URA3*
DDY196	*MAT* α *ura3-52 tpm1::LEU2 his3D200 ade2 leu2*
DDY318	*MAT* α *Dsac6::LEU2 his3D200 leu2-3,112 lys2-801 ura3-52 GAL+*
DDY322	*MAT* α *his3D200 leu2-3,112 ura3-52 Dabp1::LEU2*
DDY333	*MAT* α *his3D200 ura3-52 Dsla1::URA3*
DDY546	*MAT* α *his3D200 leu2-3,112 lys2-801 sla2-D1::URA3 ura3-52*
DDY950	*MAT* α *ura3-52 leu2-3,112 lys2-801 trp1-1 Drvs167::TRP1*
DDY952	*MAT* α *his3D200 leu2-3,112 ura3-52 lys2-801 srv2D2::HIS3*
DDY1102	*MAT* **a**/*MAT* α *ade2-1/+ his3D200/his3D200 leu2-3,112/leu2-3,112 ura3-52/ ura3-52 lys2-801/+*
DDY1266	*MAT* α *ura3-520 his3D200 leu2-3,112 lys2-801 cof1-22::LEU2*
DDY1434	MAT **a** *ade2-1 his3Δ200 leu2-3,112 ura3-52 Δtwf1::URA3*
DDY1436	*MAT* **a**/*MAT* α *ade2-1/ade2-1 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52 Δtwf1::URA3/Δtwf1::URA3*
DDY2009	MAT α *his3Δ200 leu2-3,112 lys2-801 ura3-52 pfy1-4::LEU2*
KKY62	MAT **a** *his3Δ200 ura3-52 lys2-801 cdc42-1*
MDY26	MAT **a** *lys2-801 ura3-52 leu2-3,112 his3D200 las17D::URA3*
PLY13	MAT α *ura3-52 his3D200 leu2-3,112 lys2-801 ade2-101 COF1::LEU2*
PLY15	MAT α *ura3-52 his3D200 leu2-3,112 lys2-801 cof1::LEU2*
T65.1D	MAT α *leu2-3,112 ade1 ura3-52 Ile⁻ MELI vrp1::LEU2*
YJC0388	MAT **a** *rho+ ade2-101 his3-11,15 leu2-3,112 trp1-1 ura3-1*
YJC0389	MAT **a** *rho+ ade2-101 his3-11,15 leu2-3,112 trp1-1 ura3-1 cap2-D1::HIS3*
YJC0390	MAT **a** *rho+ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cap1::TRP1*
YJC0391	MAT α *rho+ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cap1::TRP1 cap2::HIS3*

RESULTS AND DISCUSSION

4. Identification of new binding partners for twinfilin

4.1 Twinfilin localizes to cortical actin patches in yeast (I)

The localization of twinfilin in budding yeast had previously been studied using green fluorescent protein (GFP) coupled to twinfilin. This fusion protein was found to be diffusely cytoplasmic with occasional staining of cortical actin patches (Goode et al., 1998). As twinfilin localization in mammalian cells is diffuse when bound to GFP but shows cortical actin staining as an endogenous protein (Vartiainen et al., 2000), we had reason to believe that GFP disturbs twinfilin's normal localization. We therefore generated a polyclonal antibody specific for yeast twinfilin with which we investigated the localization of twinfilin in yeast cells. Endogenous twinfilin localized clearly to the cortical actin patches in yeast, in addition to some cytoplasmic staining, when detected with the affinity-purified antibody. This localization was very different from the previously obtained localization of GFP-twinfilin (Goode et al., 1998), and is likely to be due to GFP sterically hindering twinfilin´s interactions. An alternative explanation could be that the diffuse cytoplasmic localization of twinfilin seen with GFP-staining results from protein over-expression, as GFP-twinfilin expression was induced from a GAL promoter. Lack of staining in twinfilin-deleted cells confirmed specificity of the anti-twinfilin antibody.

Upon disruption of the actin cytoskeleton with the drug Latrunculin-A (Ayscough et al., 1997), twinfilin staining became diffusely cytoplasmic as the actin patches disappeared, showing the need for an intact actin cytoskeleton for correct twinfilin localization. Simultaneous studies showed that in mouse and *Drosophila*, twinfilin also localizes to cytoplasmic structures that are rich in actin monomers and filaments (Vartiainen et al., 2000; Wahlström et al., 2001), indicative of twinfilin's involvement in actin filament dynamics.

4.2 Twinfilin is an abundant protein in cells (I)

Using an antibody against twinfilin, we investigated the amount of protein present in yeast cells, by comparing known concentrations of purified proteins to different dilutions of cell extracts on a Western blot. We found twinfilin to be an abundant protein in yeast, because the actin:ADF/cofilin:twinfilin ratio in yeast cells is approximately ~ 10:2.5:1. Twinfilin has more recently been reported to be an abundant protein also in mammalian cells (Vartiainen et al., 2003), suggesting that it is well suited for regulating the size and dynamics of the actin monomer pool. In yeast, where the pool of actin monomers is reported to be smaller than in mammalian cells (Karpova et al., 1995), twinfilin could potentially sequester the majority of actin monomers at a given time. In

higher eukaryotes the significance of monomer sequestration by twinfilin could be emphasized especially in those cell-types where the actin monomer pool is limited.

4.3 Capping protein is essential for twinfilin's cellular localization (I)

Since twinfilin is an actin monomer-binding protein, it was expected to show diffuse cytoplasmic localization and not to localize to the cortical actin patches. The unexpected subcellular localization, and twinfilin's dependence on an intact actin cytoskeleton for localization, led to the search for binding partners of twinfilin on the actin filament. We examined the localization of twinfilin in a number of yeast strains with mutations in specific actin patch components. Immunofluorescence studies of these cells showed that twinfilin localized normally to the actin patches in all cells, except those in which Las17 (yeast WASP), or either subunit of the heterodimeric capping protein, was mutated. Deletion of either subunit of $\alpha 1\beta 2$ capping protein renders the protein nonfunctional (Sizonenko et al., 1996), strongly indicating capping protein as a potential binding partner of twinfilin. Actin patches are missing in the Las17 deletion mutant cells, and thus the effect of Las17 for the localization of twinfilin is most likely indirect (Li, 1997).

4.4 Twinfilin interacts with capping protein (I)

Diffuse localization of twinfilin in yeast cells lacking functional capping protein provided strong evidence for an interaction between these proteins. A native PAGE analysis using purified recombinant proteins showed twinfilin and capping protein to interact directly with each other. The two proteins also co-immunoprecipitate with one another in yeast cell extracts, indicating that they interact with each other *in vivo* as well. In addition, the twinfilin mutant Twf1-3p, which does not bind actin (see below), still interacts with capping protein on a native gel, indicating that the binding sites for actin and capping protein are located at different sites on the twinfilin molecule.

As described in the "Introduction" section, capping protein binds the barbed end of actin filaments (Cooper et al., 1999) and thereby prevents both addition and loss of actin monomers to the filament. Recent studies in yeast have provided direct evidence that the actin-capping activity of capping protein is necessary for it to function *in vivo* (Kim et al., 2004).

4.5 Twinfilin interacts with $PI(4,5)P_2$ (I, and unpublished)

Phospholipid binding and protein phosphorylation are common regulatory mechanisms for several proteins participating in cytoskeletal dynamics (reviewed in Hall, 1994; Janmey, 1994). We studied twinfilin's interaction with different phospholipids by native gel analysis

and found twinfilin to interact with PI(4,5)P_2. The effect of PI(4,5)P_2 on twinfilin's actin interactions was studied by pyrene fluorescence actin assembly assay. These studies revealed that the phospholipid decreases twinfilin's actin sequestering ability. The physiological relevance of this interaction still remains unclear, because twinfilin mutations that would specifically disrupt PI(4,5)P_2 binding, without effecting actin or capping protein binding, are not available. The fact that ADF/cofilin, profilin, twinfilin, and capping protein are all regulated by PI(4,5)P_2 indicates, however, that it is a regulatory molecule of central importance, affecting these actin binding proteins in varying ways to promote controlled treadmilling of actin filaments.

Twinfilin can be phosphorylated in mammalian cells (Vartiainen et al., unpublished), but the role of this phosphorylation for the activity of twinfilin has not been determined. The activity of yeast twinfilin does not seem to be regulated by phosphorylation, as a 2-dimensional gel-electrophoresis assay gave no indication of yeast twinfilin being phosphorylated. Similarly, ADF/cofilin, which is phosphorylated at a specific serine residue in mammals (Agnew et al., 1995), is not phosphorylated in yeast (Lappalainen et al., 1997), whereas PI(4,5)P_2 binding regulates ADF/cofilin in both yeast and mammals (Yonezawa et al., 1990).

5. Actin interactions of twinfilin

5.1 Twinfilin favors ADP-actin monomers (I)

Purified yeast and mouse twinfilin bind actin monomers in a 1:1 ratio and inhibit these monomers from polymerizing into filaments (Goode et al., 1998; Vartianen et al., 2000). Actin monomers acquire specific properties depending on the nucleotide state they are in, and thus knowing which form twinfilin preferentially binds is important for understanding its role in actin dynamics. Our native gel analysis revealed that twinfilin strongly favors MgADP-actin monomers over MgATP-actin monomers. In this regard twinfilin resembles ADF/cofilin, which also prefers actin in its "assembly-incompetent" ADP-form (Maciver and Weeds, 1994). More recent assays carried out with mouse twinfilin under physiological ionic conditions confirm twinfilin's preference for ADP-G-actin, and show it to have ~10 times higher affinity (K_d = 0.05 mM) for ADP-G-actin than for ATP-G-actin (K_d = 0.47 mM) (Ojala et al., 2002). Of the small actin monomer binding proteins that are directly involved in regulation of the actin monomer pool in cells, twinfilins and ADF/cofilins preferentially bind actin monomers in their ADP-actin form while profilins and β-thymosins prefer ATP-actin. This leads one to speculate that the ADF-H domain (Lappalainen et al., 1998), of which both ADF/cofilin and twinfilin are composed, has evolved as a domain specialized for ADP-actin binding.

5.2 Twinfilin needs bound actin for correct localization (I)

To further investigate twinfilin's localization to the actin patch, we created a twinfilin mutant unable to bind actin. To do this we took advantage of the sequence similarity between the ADF-H domains of twinfilin and ADF/cofilin, and mutated those residues shown to be most critical for ADF/cofilin's actin monomer binding (Yonezawa et al., 1990; Lappalainen et al, 1997). Our multiple sequence alignments showed these residues to be R88 and R90 in the N-terminal, and K254 and R256 in the C-terminal ADF-H domain of twinfilin. By replacing only the N-terminal domain residues with alanines we created mutant Twf1-1p, replacing the C-terminal domain residues gave us Twf1-2p, and replacing residues in both domains resulted in mutant Twf1-3p. Twf1-1p and Twf1-2p showed reduced, but not completely abolished, actin binding. Complete loss of actin binding required mutations in both domains of twinfilin, and was obtained with mutant Twf1-3p. This double mutant lost its normal subcellular localization and showed only diffuse cytoplasmic staining in yeast immunofluorescence studies, revealing that a bound actin monomer is necessary for twinfilin's localization to the actin patch.

5.3 Actin monomer binding sites are conserved between ADF/cofilin and twinfilin (I, II)

Twinfilin binds actin monomers in a 1:1 ratio (Goode et al., 1998; Vartiainen et al., 2000) although it possesses two potential actin-binding domains (ADF-H domains). The two ADF-H domains of mouse twinfilin bind actin monomers independently in a competitive manner, and the C-terminal domain shows ~ 10 times stronger actin binding than the N-terminal domain (Ojala et al., 2002). Based on kinetic data, a model explaining twinfilin's binding to actin has been proposed where the N-terminal domain of twinfilin first binds to the actin monomer, after which a conformational change within the twinfilin molecule takes place and the actin monomer is transferred to the high-affinity C-terminal domain. Additionally twinfilin competes with ADF/cofilin for actin binding, indicating that binding occurs through overlapping interfaces (Ojala et al., 2002).

The crystal structure of the N-terminal ADF-H domain of mouse twinfilin (Twf_{1-142}) was determined by Ville Paavilainen in our laboratory, at 1.6-Å resolution. The structure of Twf_{1-142} superimposed well with ADF/cofilin structures in spite of only ~ 20 % sequence homology at the amino acid level between their ADF-H domains. The regions showing highest sequence homology also showed highest structural similarity, and importantly the regions involved in actin monomer binding in ADF/cofilin were conserved in the N-terminal domain of twinfilin (figure 6). On the contrary, the regions in ADF/cofilin important for actin filament binding were very different in twinfilin (figure 6), providing structural evidence for the lack of F-actin binding in twinfilin.

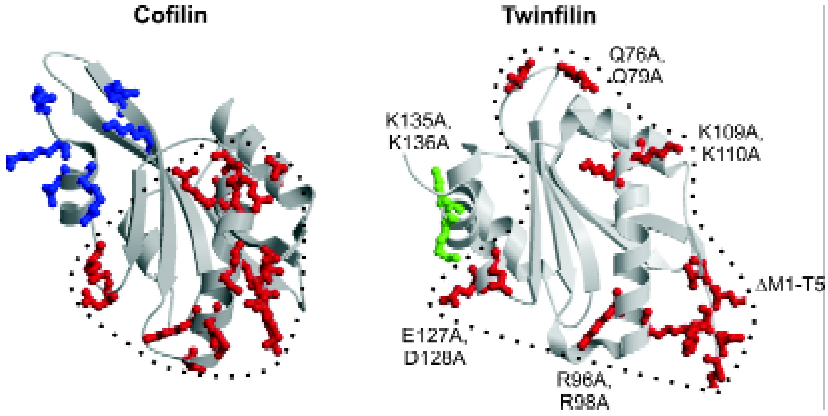

Figure 6. Actin monomer binding surfaces are conserved between ADF/cofilin and twinfilin. A ribbon diagram illustrates the structures of yeast cofilin and the N-terminal ADF-H domain of mouse twinfilin. The residues important for actin monomer binding are indicated in red, and the actin filament binding residues of ADF/cofilin are shown in blue. Twinfilin binds actin monomers similarly to ADF/cofilin, although the actin-binding surface in twinfilin is extended compared to that in ADF/cofilin. The region important for ADF/cofilin's actin filament binding is not conserved in twinfilin, and is not involved in actin binding (residues in green). Picture modified from (Paavilainen et al., 2002).

Our yeast twinfilin mutant Twf1-3p, which cannot bind actin, contains alanine substitutions in residues R88 and R90 in the N-terminal, and K254 and R256 in the C-terminal ADF-H domain (figure 7). To map twinfilin's actin monomer binding site in more detail, we introduced additional mutations in the mouse twinfilin gene. We introduced six mutations to twinfilin's N-terminal ADF-H domain at the sites important for actin binding in ADF/cofilin (figure 7). These also included the residues corresponding to those mutated in the yeast mutant Twf1-3p. The mutant proteins were expressed as GST-fusion proteins in *E. coli*. After separating the proteins from GST by trombin-digestion and purifying them by gel-filtration chromatography, we performed a urea denaturation assay for each of these mutants to confirm their stability. As all mutants showed similar overall stability to wild-type twinfilin, we determined the affinity for G-actin of every mutant protein as well as wild-type twinfilin under physiological conditions. The fluorometric NBD-G-actin binding assays revealed that five of the mutants disrupt the actin monomer binding of twinfilin, while one of the mutants ($K_{135}A$, $K_{136}A$) showed similar affinity to G-actin as wild type twinfilin. The residues mutated in this mutant ($K_{135}A$, $K_{136}A$) are located in a region of the twinfilin ADF-H domain that corresponds to the actin filament-binding region of ADF/cofilin (figure 6). Since twinfilin does not bind actin filaments, in contrast to ADF/cofilin, it is not surprising that these mutations do not interfere with its actin monomer binding. This region of twinfilin is also structurally very different compared to the corresponding region of ADF/cofilin (figure 6). The residues important for actin binding in twinfilin (figure 7) correspond well with those previously shown to be important for ADF/cofilin's actin monomer binding (Lappalainen et al., 1997), though

the actin-binding surface of twinfilin seems to be somewhat extended in comparison to the actin monomer binding region of ADF/cofilin (figure 6). Taken together, this data provides direct evidence that twinfilin and ADF/cofilin bind actin monomers through overlapping interfaces.

6. Interaction between twinfilin and capping protein

6.1 The C-terminal tail-region of twinfilin is essential for capping protein binding (III)

Capping protein is an evolutionarily conserved binding partner of twinfilin, as recently the novel mouse twinfilin isoform twinfilin-2 has also been shown to interact with capping protein. Mouse twinfilins, twinfilin-1 and twinfilin-2, interact with chicken $\alpha_1\beta_2$ and mouse $\alpha_1\beta_1$ capping proteins, showing no isoform specificity (Vartiainen et al., 2003). Mapping the site for capping protein binding on twinfilin became relevant in order to understand the mechanism of this interaction. We mapped the binding site for capping protein on yeast twinfilin by mutating evolutionarily conserved residues that, based on our previous studies, were not involved in actin monomer binding. These mutagenesis studies revealed the C-terminus of twinfilin to mediate binding to capping protein (figure 7). This binding site is evolutionarily conserved, as mutating the same region in mammalian twinfilin also abolishes capping protein binding.

We further demonstrated that the capping protein binding site is entirely located in the C-terminus of twinfilin (figure 7) since this domain alone binds capping protein, whereas the N-terminal domain shows no detectable binding to capping protein. Well in accordance with these results is the structural data of the twinfilin-capping protein complex obtained from small angle X-ray scattering studies by Ville Paavilainen in our laboratory. These data show twinfilin to be a two-domain protein, which interacts with capping protein through its C-terminal tail-region.

6.2 Twinfilin and capping protein do not affect each other's binding activities (III)

Besides interacting with capping protein, twinfilin is known to bind actin monomers and $PI(4,5)P_2$. We thus wanted to examine the ability of the yeast twinfilin mutants Twf1-10p and Twf1-11p, impaired in capping protein binding, to bind these other twinfilin ligands. To measure the affinity of Twf1-10p and Twf1-11p for ADP-actin monomers we performed NBD-assays and compared the K_d values with those of wild-type twinfilin. Wild-type twinfilin gave a K_d value of ~ 0.04 μM, Twf1-10p gave K_d ~ 0.06 μM and Twf1-11p gave K_d ~ 0.05 μM. These are all within experimental error and show that

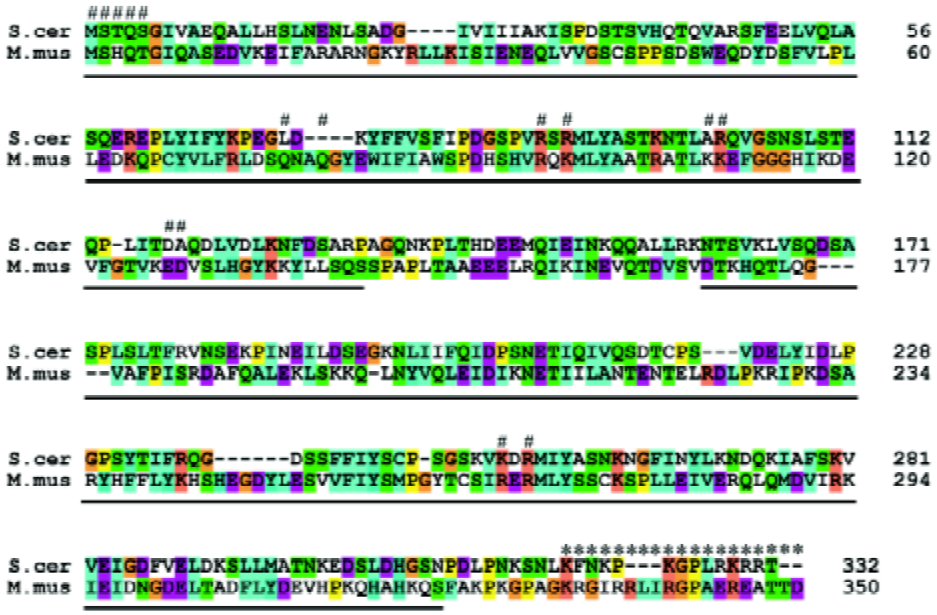

Figure 7. Sequence alignment of yeast and mammalian twinfilin, showing residues important for actin and capping protein binding. The residues important for actin monomer binding are marked with # above the sequence, while the residues shown to be important for capping protein binding are marked with *. The C-terminal tail-region of twinfilin mediates interaction with capping protein, while the actin monomer binding residues are scattered throughout the two ADF-H domains (indicated by lines below the sequences) . The alignment was generated using the program Clustal X.

the twinfilin mutants Twf1-10p and Twf1-11p bind actin monomers with equal affinity as wild-type twinfilin. To test whether twinfilin mutants Twf1-10p and Twf1-11p were still able to interact with $PI(4,5)P_2$, they were run on a native gel alone and together with $PI(4,5)P_2$. Corresponding samples containing wild-type twinfilin were run parallel on the gel, and the migration patterns compared. Both mutants as well as wild-type twinfilin showed a clear shift in motility in the presence of $PI(4,5)P_2$, demonstrating that the $PI(4,5)P_2$ binding ability of the mutants was also intact. Thus twinfilin mutants Twf1-10p and Twf1-11p fully retained their *in vitro* $PI(4,5)P_2$ binding ability as well as their ability to bind actin monomers, functioning normally except for lack of capping protein binding.

In order to better understand the interaction between capping protein and twinfilin, it was central to examine whether these proteins have any affect on each other's activities. In the laboratory of John Cooper (Washington University, St. Louis, U.S.A) a barbed end seeded actin assembly assay and a steady-state critical concentration assay were carried out to compare the ability of capping protein to cap actin filament ends in the presence and absence of twinfilin. These assays showed capping protein to inhibit barbed

end polymerization normally also in the presence of high concentrations of twinfilin, indicating that twinfilin does not regulate the activity of capping protein. Similarly, my fluorometric twinfilin-G-actin binding-assays, carried out in the presence of capping protein, showed that twinfilin's actin monomer sequestering ability is not affected by capping protein. Taken together, these data indicate that the function of twinfilin's binding to capping protein is not to regulate the activities of these proteins, but rather that twinfilin interacts with capping protein in order to be localized correctly in cells.

6.3 Twinfilin is dependent on capping protein binding for correct localization and for its role in actin dynamics (III)

To examine the biological role of the twinfilin-capping protein interaction, we expressed wild-type twinfilin, Twf1-10p, and Twf1-11p under their own promoters from *CEN* plasmids in yeast cells. As described above, these mutants specifically disrupt capping protein binding without having detectable effects on twinfilin's other known activities. The correct expression levels of these proteins were verified by Western blotting, and their *in vivo* interactions with capping protein were examined by co-immunoprecipitation assays. Twf1-10p and Twf1-11p showed no interaction with capping protein *in vivo*, and we used them to examine the relevance of this interaction for the localization of twinfilin in yeast cells. Whereas wild-type twinfilin localized to cortical actin patches in yeast cells, both Twf1-10p and Twf1-11p mutants showed diffuse cytoplasmic staining. Thus, these immunofluorescence studies revealed direct interaction between twinfilin and capping protein to be necessary for the localization of twinfilin to the cortical actin patches.

A useful method in yeast genetics is synthetic lethality, by which, depending on the nature of the starting mutation, one can uncover redundant pathways or direct protein interactions. The basic idea is that the double mutant has the lethal phenotype and is inviable, while separately the individual mutations result in viable yeast (reviewed in Forsburg, 2001). *Twinfilin* deletion yeast was previously shown to be synthetically lethal with a certain ADF/cofilin (*cof1-22*) and profilin (*pfy1-4*) mutant (Goode et al., 1998; Wolven et al., 2000). To investigate the relevance of twinfilin's interaction with capping protein, we examined whether the mutant twinfilins Twf1-10p and Twf1-11p could rescue the synthetic lethality exhibited by these cells. We generated Δ*twf1/TWF1* x *cof1-22/COF1* and Δ*twf1/TWF1* x *pfy1-4/PFY1* diploid yeast strains and transfected these with empty *CEN* plasmids or plasmids containing wild-type *TWF1* or *twf1-10* genes. These yeasts were then sporulated, their tetrads dissected, and the resulting growth was analyzed on different selection media. The construct containing wild-type *TWF1* could, as expected, rescue the synthetic lethality of both Δ*twf1* x *cof1-22* and Δ*twf1* x *pfy1-4* yeast cells. The mutant twinfilin, *twf1-10*, was unable to rescue the synthetic lethality exhibited by these cells, as was the empty plasmid lacking the *TWF1*

gene. After prolonged (> 5 days) incubation on plates, very small colonies occasionally appeared also of the Δ*twf1 cof1-22* and *twf1-10 cof1-22* strains. Immunofluorescence experiments showed that in both cases the phenotypes were virtually identical, with abnormally large cell-size and severely disrupted actin cytoskeleton. Taken together, these data demonstrate that twinfilin is dependent on its capping protein binding ability to perform its role in actin dynamics *in vivo*.

7. The role of twinfilin in actin dynamics (I, III, IV)

The first studies of yeast and mammalian twinfilins (Goode et al., 1998; Vartiainen et al., 2000) gave some indications of a possible role in the regulation of actin dynamics. Few conclusions concerning twinfilin´s precise function in cells could, however, be made based upon the data available at that time. Twinfilin was known to bind actin monomers in yeast and in mammalian cells in a 1:1 ratio, but not to interact with actin filaments. The localization of GFP-twinfilin was diffusely cytoplasmic in both yeast and mammalian cells, but antibody staining of mammalian twinfilin revealed strong cytoplasmic staining indicating that the GFP had disturbed twinfilin's normal localization (Goode et al., 1998; Vartiainen et al., 2000). The localization to cortical structures, seen by antibody staining of twinfilin in mammalian cells, was a strong indication of twinfilin's involvement in actin dynamics (Vartiainen et al., 2000). Also, the fact that twinfilin was shown to inhibit nucleotide exchange on the actin monomer after binding, together with the phenotypes obtained with twinfilin yeast mutants (Goode et al., 1998), pointed towards twinfilin being a novel actin-sequestering protein involved in the regulation of cytoskeletal dynamics.

Based on the data obtained in this study, together with other recent data, we are now able to present a "mailman model" (Palmgren et al., 2002; see also figure 8) that provides a plausible explanation for twinfilin's role in cells as a localizer of actin monomers. This model postulates that once an actin monomer has been depolymerized from the pointed end of an actin filament by ADF/cofilin (Carlier et al., 1997), twinfilin can take this monomer from ADF/cofilin (figure 8). Twinfilin has been shown to compete with ADF/cofilin for actin monomer binding (Ojala et al., 2002), supporting such an idea. After binding the actin monomer with its N-terminal ADF-H domain, a conformational change in twinfilin would allow the monomer to be moved to the C-terminal domain, which exhibits stronger actin binding affinity (Ojala et al., 2002). As we have shown twinfilin to preferentially bind ADP-actin monomers, it could now localize the monomer in its "inactive" ADP-form to sites in the cell where actin polymerization takes place (figure 8). Antibody staining of yeast twinfilin revealed it to localize to cortical actin patches in yeast cells, which are places of high filament turnover. Our results also show

twinfilin to be an abundant protein in cells, which means that it can sequester a large proportion of the actin monomer pool at any given time. Combined with twinfilin hindering nucleotide exchange upon binding actin (Goode et al., 1998), its effect on the actin monomer pool could be significant. For correct localization in yeast cells, we have shown twinfilin to directly interact with capping protein at the barbed end of the actin filament (figure 8). In addition, we have shown that this interaction is crucial for twinfilin's function in cells, indicating that the role of this interaction could be to deliver actin monomers for filament polymerization (figure 8).

Slightly confusing is that twinfilin interacts with capping protein and thus localizes to capped filaments instead of filaments that are ready for elongation. Capping protein binds the barbed end of actin filaments and prevents the addition and loss of actin monomers to the filament end (Wear et al., 2003; Kim et al., 2004). It is important to note, however, that in yeast cells the tiny size of actin patches probably means that capped filaments are located in very close vicinity to uncapped ones, allowing monomers at capped filaments to rapidly diffuse to uncapped filament barbed ends close by. We have shown that mouse twinfilin also interacts with capping protein, but wheter this interaction is required for the localization of twinfilin in mammalian cells remains to be investigated. After the monomer has been released from twinfilin, profilin could mediate nucleotide exchange on the monomer before its incorporation into an elongating filament (figure 8).

Theoretically, release of the actin monomer bound to twinfilin could occur by a conformational change occurring in twinfilin upon binding to capping protein at the filament end. Our studies showed that capping protein does not influence twinfilin's actin monomer binding ability, but as these experiments were carried out using soluble capping protein, it might not reflect the situation at the barbed end of the filament. The fact that capping protein undergoes a conformational change upon binding to the actin filament (Yamashita et al., 2003; Wear et al., 2003), combined with the location of twinfilin's high affinity actin binding site close to its C-terminal capping protein binding site, indicates that a conformational change in capping protein could result in the release of the actin monomer from twinfilin. Another possibility is that some currently unidentified protein interacts with the twinfilin-actin-capping protein complex at the barbed end of the filament mediating release of ADP-G-actin from twinfilin. As these mechanisms account only for the monomer release from twinfilin, they do not explain how capping protein could potentially be removed from the filament end. $PI(4,5)P_2$ inhibits actin binding both of capping protein (Amatruda and Cooper, 1992) and twinfilin, allowing for the theoretical possibility that this second messenger regulates barbed end elongation by interaction with these two proteins. $PI(4,5)P_2$ binding to capping protein releases capping protein from the filament barbed end (Schafer et al., 1996) and its simultaneous binding to twinfilin

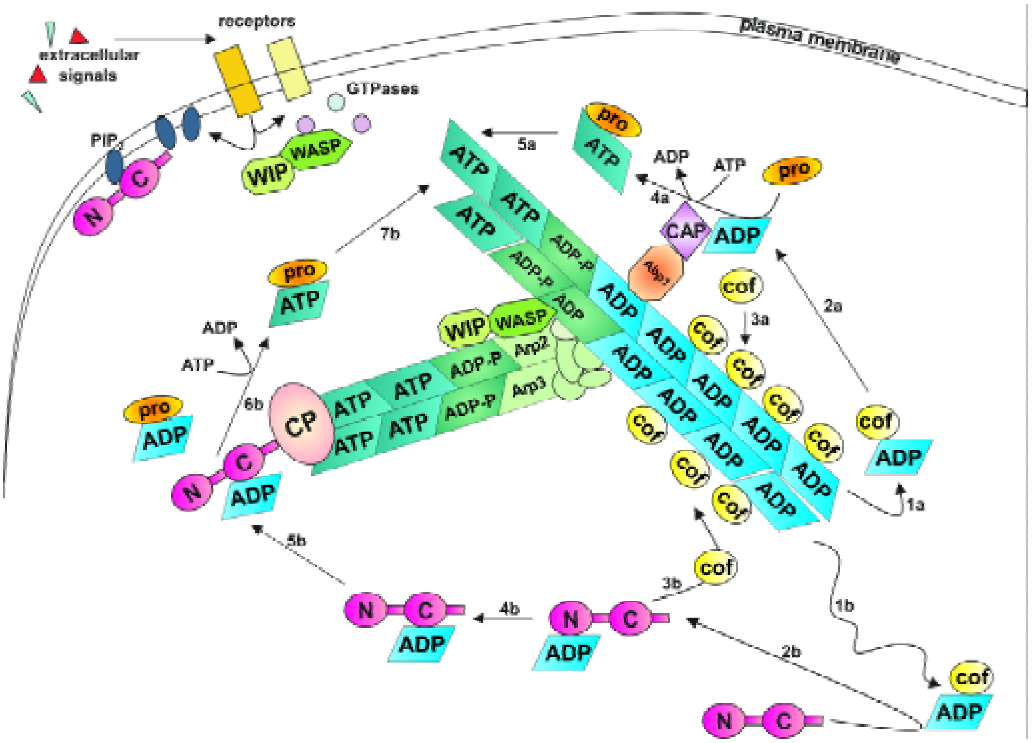

Figure 8. Hypothetical model illustrating twinfilin's function in cells as a protein that sequesters actin monomers from unwanted places, and delivers them to sites of actin polymerisation. *Pathway a: Recycling of actin monomers by Srv2/CAP.* ADF/cofilin depolymerizes ADP-G-actin from the pointed end of the actin filament (1a). The ADF/cofilin-actin complex then interacts with Srv2/CAP, bound to Abp1 at the plasma membrane, and Srv2/CAP takes the actin monomer from ADF/cofilin (2a). ADF/cofilin is thereby released for a new round of actin depolymerization (3a). Profilin then catalyzes the exchange of actin nucleotide on the actin monomer (4a), and the ATP-actin monomer is thus ready for incorporation into the filament (5a). *Pathway b: Localization of ADP-G-actin by twinfilin.* An actin monomer that ADF/cofilin has depolymerized from the pointed end of the actin filament diffuses further away from the actin patch (1b). Twinfilin catches the ADF/cofilin bound ADP-actin monomer from ADF/cofilin at an unwanted place in the cell (2b), releasing ADF/cofilin for further actin depolymerization (3b). Twinfilin binds the actin monomer first with its low affinity N-terminal domain, but soon shifts the actin monomer to its high affinity C-terminal domain (4b). Twinfilin then transports the actin monomer in its "inactive" ADP-form to places in the cell where filament polymerisation is taking place. This localization is promoted by interaction between twinfilin's C-terminal tail-region and capping protein (5b). Profilin then possibly takes the actin monomer from twinfilin, exchanges its nucleotide to ATP (6b) and mediates the incorporation of the ATP-G-actin into the barbed end of the filament (7b). PI(4,5)P_2 at the plasma membrane binds twinfilin and inhibits its actin monomer binding. Thus "pathway a" illustrates normal recycling of actin monomers by Srv2/CAP, while "pathway b" illustrates how twinfilin hinders actin monomers from polymerizing in unwanted places in the cell by transporting them to places where actin polymerisation is taking place.

could result in release of the actin monomer, providing both a free actin filament end and a free actin monomer for elongation. Whether this speculative mechanism will prove to be correct remains to be seen.

Although our studies suggest that twinfilin is involved in recycling actin monomers from ADF/cofilin to new rounds of assembly, other proteins also appear to contribute to this aspect of actin dynamics. The fact that the actin-ADF/cofilin complex has been indicated to be a target also for Srv2/CAP (Moriyama and Yahara, 2002) suggests that partially overlapping functions must exist for twinfilin and Srv2/CAP. Srv2/CAP has been shown to displace ADF/cofilins from actin monomers, thereby releasing ADF/cofilin for further depolymerization and simultaneously freeing actin monomers for polymerization (Moriyama and Yahara, 2002; Balcer et al., 2003). Comparison of the known properties of these two proteins, however, indicates that Srv2/CAP is involved in recycling of actin monomers locally at actin patches, while twinfilin could have a more specialized function in actin monomer localization (figure 8).

It is important to note that hindering uncontrolled polymerization from occurring in the wrong places in cells is of equal importance as structured polymerization occurring in a coordinated manner. Twinfilin is well suited to function as a protein that binds actin monomers located in undesired places in the cell, transport them to sites of actin polymerization, and thereby hinder polymerization from occurring in unwanted sites. Support for this type of model (figure 8), where twinfilin's function is to ensure delivery of actin monomers to the correct sites in the cell, comes from studies in *Drosophila*. The punctuate localization of twinfilin in fully elongated *Drosophila* bristles may represent localization to the barbed ends of actin filaments, indicating that also in these cells twinfilin serves to localize actin monomers to sites of actin filament assembly. *Drosophila* bristles lacking functional twinfilin contain a large number of missoriented and misslocalized actin filament structures, indicating that spontaneous uncontrolled actin nucleation takes place in these cells (Wahlström et al., 2001).

In spite of some remaining open question about the precise mechanism by which twinfilin functions, it is well established by now that twinfilin belongs to the central, conserved, ubiquitous proteins involved in regulation of actin dynamics. Twinfilin is one of the central pieces in this complex puzzle of actin dynamics, but exactly how all the pieces fit together remains to be seen. Completion of the puzzle is made all the more challenging due to the fact that a number of pieces are still likely to be missing.

CONCLUSIONS AND FUTURE PROSPECTS

This thesis work has revealed many of the basic properties of twinfilin, and based on this information a plausible model for twinfilin's function in cells is suggested (see previous section and figure 8). Based on this work we now know twinfilin to be an abundant protein that localizes to cortical actin patches in yeast cells. Twinfilin interacts with ADP-G-actin and capping protein, and these interactions are crucial for its correct cellular localization. Additionally, $PI(4,5)P_2$ is shown to bind twinfilin, thereby hindering actin monomer binding. The interaction of twinfilin with capping protein was studied in detail, revealing twinfilin to bind capping protein with its C-terminal tail-region and demonstrating this interaction to be vital for twinfilin's function in cells.

Nonetheless, several unanswered questions remain regarding our model for twinfilin's function in cells (figure 8). Some major questions concerning this model are: How does twinfilin release its bound actin monomer upon binding to capping protein? How does capping protein allow monomer incorporation, if indeed it does so at all? If not, how does the monomer reach an uncapped actin filament barbed end? And also, why does twinfilin possess two ADF-H domains although it binds only one actin monomer? Is there still some unknown ligand for twinfilin?

A possible method to search for other potential ligands for twinfilin would be a yeast-two hybrid screen. A novel ligand might also provide an answer for how the monomer is released from twinfilin and incorporated into a growing actin filament. More knowledge of the *in vivo* importance of $PI(4,5)P_2$ binding to twinfilin and capping protein might also provide clues as to how the actin monomer moves from twinfilin, past capping protein, into the growing actin filament. Unfortunately studying complex processes involving several components, as in this case twinfilin, actin monomer, actin filament, capping protein and possibly also $PI(4,5)P_2$, has its own problems and there are no obvious straightforward assays or solutions. Nevertheless, future research will certainly provide answers to the open questions regarding release and incorporation of the actin monomer transported by twinfilin. Crystal structures of twinfilin's C-terminal domain as well as twinfilin in complex with actin and/or capping protein would also provide useful answers to these protein interactions, and as to whether a conformational change mediates the release of the actin monomer from twinfilin.

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